Stability of a Novel Hexapeptide, (Me)Arg–Lys–Pro–Trp–*tert*-Leu–Leu–OEt, with Neurotensin Activity, in Aqueous Solution and in the Solid State

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The stability and some physicochemical properties of a novel hexapeptide, (Me)Arg–Lys–Pro–Trp–*tert*-Leu–Leu–OEt (I), with neurotensin activity, were investigated. The degradation of I in aqueous solution was observed as a pseudo-first order reaction. By determining the degradation rate of I at various pH values, it was found that I was most stable at around pH 4. The activation energies of the degradation in aqueous solutions at pH 2.2, 6.1, 7.0 and 8.0 were 16.3, 22.2, 23.9 and 24.2 kcal/mol, respectively.

The enzymatic hydrolysis of I was studied *in vitro* with a porcine liver esterase at 37 °C. The degradation of I in this system was observed as a pseudo-first order reaction. The degradation rate of I in the presence of the esterase was about 10000 times larger than the rate in a buffer solution.

I in the solid state was stable under 65 °C and labilized by strong light and/or high humidity. The pK_{a1}, pK_{a2} and pK_{a3} of I were 7.1, 10.0 and 11.3, respectively. The partition coefficients between *n*-octanol and the buffer solution at pH values ranging from 2 to 11 were measured. The partition coefficient increased with the increase of the pH value. But the value at pH 7.0 was 2.10×10^{-2}, which was very low. The solubility of I in aqueous solution was more than 10 mg/ml. From the results of the powder X-ray diffraction pattern, I in the solid state was found to be amorphous. The dissolution rates in the 1st and 2nd fluid of JP XI at 37 °C and 100 rpm were 19.4 and 9.0 mg/cm^2 · min, respectively.

Keywords hexapeptide; neurotensin activity; degradation rate; aqueous solution; solid state; esterase; activation energy; pK_a; dissolution rate; partition coefficient

(Me)Arg–Lys–Pro–Trp–*tert*-Leu–Leu–OEt (I) is our new synthetic hexapeptide. I is an ethyl ester form of the hexapeptide, (Me)Arg–Lys–Pro–Trp–*tert*-Leu–Leu (II). These hexapeptides are confirmed to have approximately equal neurotensin activity based on the effects of I and II on methamphetamine-induced hyperactivity in rats.[1] It has been suggested that neurotensin is closely associated with the dopaminergic neurons, particularly in the mesolimbic system, and that one of the physiological roles of neurotensin is the modulation of dopaminergic neurons.[2–4] Accordingly, I has possibilities as a new type of psychotropic drug.

In order to select a route of administration and design a dosage form for a new compound from the standpoint of the optimization of drug delivery, it is necessary to evaluate its physicochemical, pharmacokinetic and pharmacodynamic properties exactly. Thus, we began to investigate the physicochemical properties as part of a series of studies on the pharmaceutical evaluation of I. In this report, we described the stability of I in an aqueous solution and in solid state under various conditions, and in addition, its stability in the presence of esterase, its solubility, partition coefficient, pK_a and dissolution rate.

Experimental

Materials (Me)Arg–Lys–Pro–Trp–*tert*-Leu–Leu–OEt·3HCl (I), the molecular weight of which is 963.49, was synthesized in our laboratories by conventional methods in solution using the mixed anhydride coupling procedure.[1] It was deprotected and purified by means of low performance liquid chromatography (LPLC) on CM-TOYOPEARL (carboxymethylcellulose) followed by high performance liquid chromatography (HPLC). I was checked for purity by HPLC and confirmed to be more than 98% pure. Other chemicals were of reagent grade.

Kinetic Measurement of Degradation of I in Aqueous Solution I was dissolved in buffers to make a 0.25 mg/ml solution. The pH values of the Britton-Robinson buffer (1/15 M, $\mu=0.5$)[5] used were as follows; pH 2.2, 3.0, 4.2, 5.1, 6.1, 7.0 and 8.0. The pH values of these buffer solutions were checked before and after the reaction, and remained unchanged. A 5 ml aliquot of the buffer solution containing 0.25 mg/ml of I was placed in a 10 ml glass ampule, which was sealed and kept in a controlled-temperature oven (Yamato Scientific Co., Ltd., Tokyo, Japan) at 45, 50, 60 and 80 °C with 0.1 °C precision. The ampules were withdrawn at appropriate time intervals and cooled to room temperature. 50 μl of an internal standard solution containing 0.1 mg/ml of *n*-propyl-*p*-hydroxybenzoate in ethanol and 500 μl of 0.02 M HCl was added to 200 μl of the solution taken from the ampule. A 20 μl dose of the mixture was injected onto the HPLC column.

Determination of I by HPLC The HPLC system consisted of a Shimadzu model LC-6A pump, SIL-6B auto-injector, SPD-6A UV photometrical detector and CTO-6B column oven and equipped with a Shimadzu model SCL-6B system controller and C-R4AX chromatopac. The chromatographic column was a YMC Pack A3027 ODS (150 × 4.6 mm i.d.) obtained from Yamamura Chemical Lab., Co., Ltd. (Kyoto, Japan). The flow rate, the wavelength for determination and the temperature of the column were 1 ml/min, 280 nm and 40 °C, respectively. The mobile phase used for the determination of I was acetonitrile–water–$HClO_4$ (60%), 350 : 650 : 1. The capacity factor, k', was measured using mobile phases containing different concentrations of acetonitrile from 28% to 40% and 0.1% $HClO_4$. The column dead time, t_0, was determined using cytosine as the non-retained compound. The capacity factor, k', is defined as:

$$k' = (t_r - t_0)/t_0 \quad (1)$$

where t_r is the retention time of the compound.

To make a calibration curve for I, standard solutions spiked at the 2.5, 7.5, 12.5, 25, 75, 125, 175 and 250 μg/ml levels in 0.1 N HCl were prepared. 50 μl of the internal standard solution and 500 μl of 0.02 M HCl were added to 200 μl of each standard solution. A 20 μl sample of the mixture was injected onto the HPLC column. The calibration curve was constructed by plotting the peak area ratio of I to the internal standard *versus* the standard concentration.

Stability Measurements with the Esterase System The hydrolytic rate of I in the presence of esterase was determined according to the method reported by Kawaguchi *et al.*[6]

A partially purified porcine liver esterase (Sigma E-3128) was used. A 1 ml aliquot of the esterase suspension (in 3.2 M $(NH_4)_2SO_4$ solution, 2860 units/ml) was diluted with 14.3 ml of 0.1 M pH 7.00 phosphate buffer; then, this solution was filtered through a membrane filter (Gelman Science, 0.2 μm), and used for the experiments. The hydrolytic rate of I was determined in the presence of the esterase preparation diluted appropriately with an isotonic phosphate buffer (pH 7.00) containing 0.19 M sucrose. The experiments were performed at 37 °C and initiated by adding the I solution (3.85 mg/ml, 4×10^{-3} M) to produce a final concentration of 4×10^{-5} and 7.8×10^{-5} M. The decrease of I was followed by HPLC analysis of samples taken periodically from the reaction mixture.

Stability in the Solid State A sample of compound I (5 mg) was packed in a light-resistant glass vessel, except for the sample placed on open Petri disks in the light cabinet. The samples were stored under conditions as follows: 40, 45, 55, 65 and 80 °C, 40 °C × 75% R. H., and 1000 lux, for different intervals of time, and then analyzed by HPLC.

pK_a and Dissolution Rate The pK_a value was determined by a titration method.

The dissolution rate of I from a constant surface area of 1 cm^2 was studied using an apparatus designed by Wood *et al.*[7] About 200 mg of I was compressed by a Shimadzu hydraulic press for KBr tablets for infrared (IR) spectroscopy to form a pellet at 1500 kg of pressure. The compressed disk was not ejected from the die, and the die carrying the compressed disk was set on the dissolution apparatus so that the disk face became the stirrer. A 200 ml sample of the 1st fluid or 2nd fluid of JP XI was used as the dissolution medium, and the temperature of the medium was maintained at 37±0.5 °C. The disk was rotated at 100 rpm. 200 μl of the solution was taken at appropriate time intervals, and the concentration was determined by HPLC assay.

Powder X-Ray Diffraction Study Powder X-ray diffractometry was carried out using a Rigaku Denki Geigerflex model RAD-1B with Ni-filtered Cu-K_α radiation.

Solubility A 10 mg sample of I was added to 1 ml of various solvents or buffer solutions in a centrifuge tube, which was then sealed and shaken at 25 °C for 30 min. After being centrifuged for 20 min at 2500 rpm, 10 μl of the supernatant was injected into the HPLC, and the concentration was determined.

Partition Coefficient The partition coefficient of I was determined in an *n*-octanol–Britton-Robinson buffer system according to the method of Kakemi *et al.*[7]

I was unstable in a basic aqueous solution, so the determination of the partition coefficient was performed at 25 °C. The half-life of I in the buffer solution of pH 11 at 25 °C was estimated to be about 5 h. The determination of the partition coefficient of I in the buffer solution of pH 11 was judged to be possible from the half-life. Therefore, the partition coefficient of I was determined in the range of pH 2 to 11.

Results and Discussion

Determination of I by HPLC I and II can be determined by HPLC. Table I shows the effect of acetonitrile concentration in the mobile phase on the capacity factors of I, II and *n*-propyl-*p*-hydroxybenzoate. From this result, the mobile phase, acetonitrile–water–$HClO_4$, 350 : 650 : 1, was used for the determination of I. The retention times of I, II and *n*-propyl-*p*-hydroxybenzoate were 5.3, 2.5 and 10.2 min, respectively. The detection limit of I is estimated to be 0.7 μg/ml with a signal-to-noise ratio of *ca.* 10 : 1. The calibration curve is linear from 2.5 to 250 μg/ml. The relationship between the concentration of I and the peak-area ratio is expressed as $y=0.01011x-0.00883$, where x is the concentration of I (μg/ml) and y is the ratio of peak-area. The correlation coefficient (r) is 0.99999. The intraday accuracy and precision of this method, as shown in Table II, produced acceptable results. The interday variation at 250 μg/ml calculated from the variation of peak-area ratio was 2.2% ($n=7$).

TABLE I. Effect of Acetonitrile Concentration in the Mobile Phase on the Capacity Factors (k') of I, II and *n*-Propyl-*p*-hydroxybenzoate

Concentration of acetonitrile (%, v/v)	Capacity factor (k')		
	I	II	*n*-Propyl-*p*-hydroxybenzoate
28	12.44	2.19	12.21
30	7.43	1.44	9.65
32	4.62	0.98	7.65
35	2.44	0.59	5.60
40	1.00	0.27	3.59

Degradation Products in Aqueous Solution I was hydrolized to II. These peptides were confirmed by the retention time of HPLC. II was not degraded under the conditions used for measuring the kinetics of degradation of I in aqueous solution. Therefore, the degradation product of I in aqueous solution is estimated to be just II.

Stability in Aqueous Solution Typical time courses of the disappearance of I at various pH values at 80 °C, and at various temperatures at pH 8.0 are shown in Figs. 1 and 2, respectively. It is obvious that the degradation followed an overall pseudo-first order kinetics pattern with respect to I. The pseudo-first order rate constants, k_{obs}, were calculated from the slopes of semilogarithmic plots of the residual percent against time. The pseudo-first order rate constants (k_{obs}) are shown in Table III.

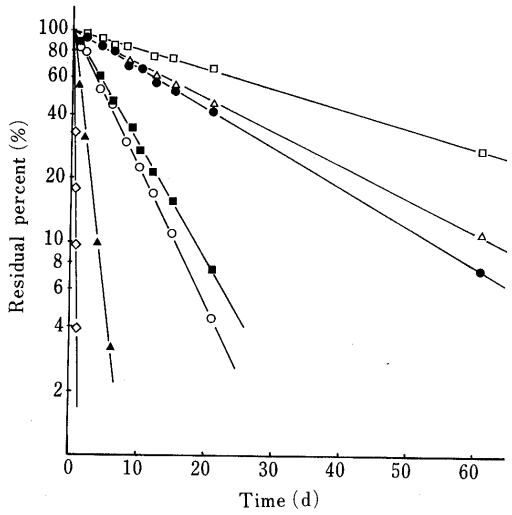

Fig. 1. First-Order Plots for the Degradation of I in Buffer Solutions of pH 2.2—8.0 at 80 °C

○, pH 2.2; ●, pH 3.0; □, pH 4.2; △, pH 5.1; ■, pH 6.1; ▲, pH 7.0; ◇, pH 8.0.

TABLE II. Accuracy and Precision of the Determination of I

Actual concentration (μg/ml)	Peak-area ratio (mean±S.D., $n=5$)	Concentration found (mean±S.D.)	Coefficient of variation (%)	Accuracy (%)
2.5	0.0156±0.0014	2.413±0.141	5.8	96.5
7.5	0.0675±0.0024	7.552±0.234	3.1	100.7
12.5	0.1161±0.0018	12.352±0.177	1.4	98.8
25	0.2394±0.0023	24.544±0.231	0.9	98.2
75	0.7509±0.0021	75.121±0.211	0.3	100.2
125	1.2663±0.0097	126.037±0.950	0.8	100.9
175	1.7597±0.0172	174.878±1.697	1.0	99.9
250	2.5149±0.0243	249.549±2.398	1.0	99.8

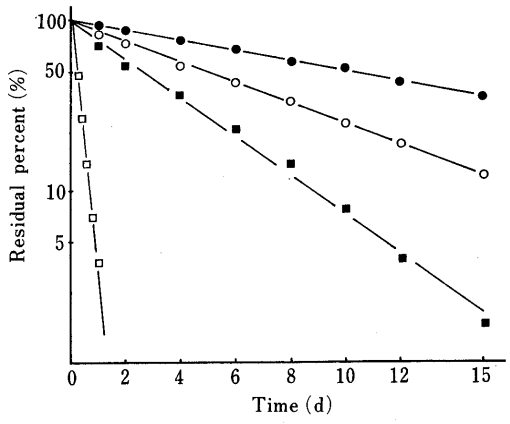

Fig. 2. First-Order Plots for the Degradation of I in an Aqueous Solution of pH 8.0

●, 45 °C; ○, 50 °C; ■, 60 °C; □, 80 °C.

TABLE III. Pseudo-First Order Rate Constants for the Degradation of I in Buffer Solutions

pH	°C	k_{obs} (d^{-1})
2.2	45	1.09×10^{-2}
	50	1.85×10^{-2}
	60	3.17×10^{-2}
	80	1.50×10^{-1}
3.0	45	—
	50	—
	60	2.60×10^{-3}
	80	4.27×10^{-2}
4.2	45	—
	50	—
	60	2.60×10^{-3}
	80	2.09×10^{-2}
5.1	45	—
	50	2.07×10^{-3}
	60	2.69×10^{-3}
	80	3.58×10^{-2}
6.1	45	3.90×10^{-3}
	50	6.04×10^{-3}
	60	1.11×10^{-2}
	80	1.28×10^{-1}
7.0	45	1.23×10^{-2}
	50	2.56×10^{-2}
	60	4.99×10^{-2}
	80	5.71×10^{-1}
8.0	45	7.04×10^{-2}
	50	1.25×10^{-1}
	60	2.66×10^{-1}
	80	3.23

The k_{obs} values were not obtained under the following conditions: pH 3.0 at 45 and 50 °C, pH 4.2 at 45 and 50 °C, and pH 5.1 at 45 °C, because I was fairly stable under those conditions. The residual percents of I under those conditions after 61 d are shown in Table IV.

Figure 3 shows the relationship between the pseudo-first order rate constant (k_{obs}) and pH at 80 °C on the basis of the data shown in Table III. It was found that the degradation rate of I was influenced by the pH of the medium, and that I was most stable in a buffer solution of around pH 4.

The obtained k_{obs} values at 60 °C and 80 °C in the pH 4.2 buffer solution were 2.60×10^{-3} and $2.09\times10^{-2}\,d^{-1}$, respectively. It was reported that nafareline acetate, an analog of luteinizing hormon releasing hormon (LH-RH), was degraded in aqueous solution, and that the $\log k_{obs}$ *vs.* pH profile at 80 °C displayed a U-shaped profile with a pH region of maximum stability between pH 4 and 6.[8] The k_{obs} of nafareline acetate at 80 °C in the pH 4.54 buffer solution is $1.7\times10^{-5}\,s^{-1}$ ($1.47\times10^{-2}\,d^{-1}$). The obtained k_{obs} value of I was nearly equal to that of nafareline acetate at around pH 4. The degradation rate constants (k_{obs}) of secretin at 60 °C presented by Tsuda *et al.*[9] are from 1×10^{-1} to $1\times10^{-2}\,h^{-1}$ in the region from pH 2 to 8. Compared with the k_{obs} values of secretin, the degradation rate of I is considered to be fairly slow except at pH 8.0. Therefore, I can be regarded as a fairly stable peptide in aqueous solution.

TABLE IV. Residual Percents of I in Buffer Solutions after 61 d

pH	°C	%
3.0	45	96.0±3.0
	50	94.1±2.5
4.2	45	96.0±3.0
	50	94.1±2.5
5.1	45	95.0±0.8

Each value represents the mean±S.D. of three determinations.

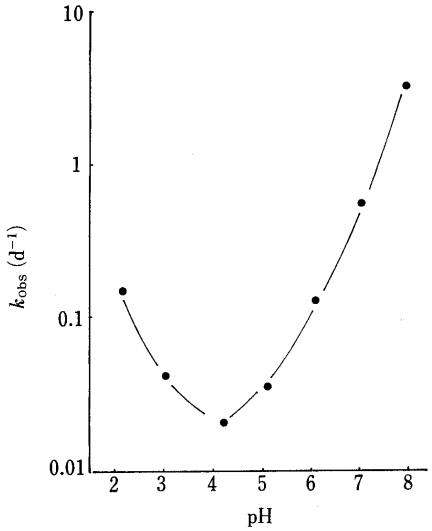

Fig. 3. pH-Rate Profile for the Degradation of I in the Buffer Solution at 80 °C

TABLE V. Activation Energy of Degradation of I

pH	E_a (kcal/mol)
2.2	16.3
6.1	22.2
7.0	23.9
8.0	24.2

The pseudo-first order rate constants (k_{obs}) as shown in Table III were increased with increasing temperature at predetermined overall pH points.

The activation energies of the reaction at pH 2.2, 6.1, 7.0 and 8.0 were calculated from the Arrhenius plots, and are summarized in Table V. The activation energy of I at pH 6.1 was nearly equal to that of nafareline acetate at pH 5.4,

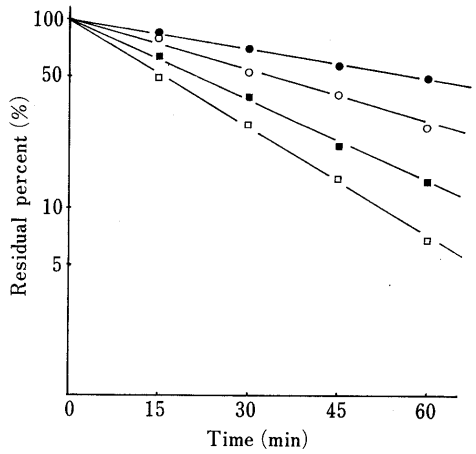

Fig. 4. Porcine Liver Esterase-Catalyzed Hydrolysis of I at 37 °C
●, 2.5 units; ○, 5.0 units; ■, 7.5 units; □, 10.0 units.

TABLE VI. Effects of Esterase Concentration and Substrate Concentration on Enzymatic Hydrolysis Rates of I

Substrate concentration ($\times 10^{-5}$ M)	Enzyme concentration (unit/ml)	k_{obs} (min^{-1})
4.0	2.5	1.21×10^{-2}
	5.0	2.11×10^{-2}
	7.5	3.25×10^{-2}
	10.0	4.53×10^{-2}
7.8	2.5	1.10×10^{-2}

TABLE VII. Stability of I in the Solid State

Condition	Stored period	Residual percent
40 °C	1 M	99.1
	2 M	99.3
	3 M	97.4
45 °C	1 M	99.2
	2 M	99.5
	3 M	98.4
55 °C	1 M	98.4
	2 M	97.3
	3 M	97.1
65 °C	1 W	100.0
	2 W	99.3
	3 W	97.6
	1 M	98.2
80 °C	1 W	97.6
	2 W	92.2
	3 W	86.5
	1 M	83.4
40 °C × 75% R.H.	2 W	98.9
	3 W	96.3
	1 M	96.6
1000 lux	2 W	98.4
	3 W	86.3
	1 M	90.2

M, month; W, week.

which was reported to be 21 kcal/mol.[8]

Enzymatic Hydrolysis The enzymatic hydrolysis of I was studied *in vitro* with porcine liver esterase at 37 °C. The degradation product in this system was confirmed to be only II by HPLC assay. The time courses of the disappearance of I at various esterase concentrations are shown in Fig. 4. The slopes of semilogarithmic plots of the residual percent against time produced pseudo-first order rate constants. Table VI summarizes the effect of the esterase concentration and substrate concentration on the hydrolysis rate of I. The degradation rate constants showed a significant dependence on enzyme concentration, while the rate constants were not decreased at a higher substrate concentration.

It was difficult to determine the degradation rate of I in the buffer solution at pH 7.0 at 37 °C in a short period, so the rate constant was calculated from the activation energy at pH 7.0 as shown in Table V. The calculated rate constant was 3.15×10^{-6}, which was about 1/10000 compared with the enzymatic hydrolysis rate constants. This indicates that I is stable chemically, but very unstable in the presence of esterase. Therefore, further investigation of the enzymatic hydrolysis of I will be required.

Stability in the Solid State The stability of I in the solid state was shown in Table VII. This result indicates that I is stable under 65 °C, and is unstable under strong light and/or high humidity. There is some illogical data in Table VII; for example, the residual percent of 3 weeks under 1000 lux is smaller than that of 1 month. These results are

TABLE VIII. Partition Coefficients of I between *n*-Octanol and Buffer Solutions

pH of buffer	Partition coefficient
2	1.58×10^{-3}
3	2.18×10^{-3}
4	2.56×10^{-3}
5	5.47×10^{-3}
6	5.59×10^{-3}
7	2.10×10^{-2}
8	6.50×10^{-2}
9	2.71×10^{-1}
10	7.18×10^{-1}
11	8.96×10^{-1}

TABLE IX. Solubility of I

Solvent	Solubility (mg/ml)
Methanol	>10
Ethanol	>10
n-Butanol	9.6
2-Propanol	5.3
Polyethylene glycol 400	>10
Propylen glycol	>10
Acetonitrile	<0.0005
Acetone	<0.0005
Chloroform	0.1
Dichloromethane	0.1
Diethyl ether	<0.0005
Ethyl acetate	<0.0005
Benzene	0.009
Hexane	<0.0005
Distilled water	>10
The 1st fluid of JP XI	>10
The 2nd fluid of JP XI	>10
Buffers pH 2	>10
pH 3	>10
pH 4	>10
pH 5	>10
pH 6	>10
pH 7	>10
pH 8	>10

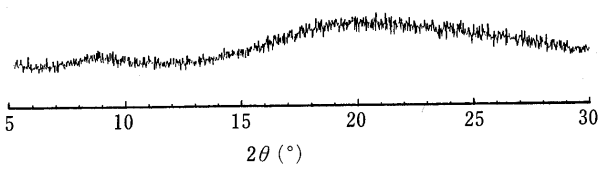

Fig. 5. Powder X-Ray Diffraction Patterns of I

considered to be caused by experimental error.

pK_a, Partition Coefficient and Solubility The pK_a values, pK_{a1}, pK_{a2} and pK_{a3}, were 7.1, 10.0 and 11.3, respectively.

Table VIII shows the partition coefficients of I between *n*-octanol and a buffer solution of various pH values. The partition coefficient increased with an increase of pH values. But the values were very low. For example, at pH 7.0 and 11.0 they were 2.10×10^{-2} and 8.96×10^{-1}, respectively.

The solubility of I in the various solvents is shown in Table IX.

Powder X-Ray Diffraction Pattern and Dissolution Rate Figure 5 shows the powder X-ray diffraction pattern of I. No peak was observed. This result indicates that I in the solid state is amorphous.

The dissolution rates in the 1st and 2nd fluids of JP XI were 19.4 and 9.0 mg/cm^2·min, respectively. It is considered that these large values are caused by the high solubility of I in aqueous solution (>10 mg/ml) and also its amorphous shape. The difference of the rate between the 1st and 2nd fluids is presumed to be caused by the difference of solubility affected by the pK_a value.

As noted above, a novel hexapeptide, I, is chemically stable in an aqueous solution of around pH 4 and in the solid state except under the conditions of strong light and high humidity. These results suggest that it is possible to prepare a solution dosage form for injection and a solid dosage form for oral administration. However, the degradation rate of I in the presence of porcine liver esterase was very rapid. Further study with other enzyme systems is essential to consider the administration route, dosage form design and analysis of the pharmacokinetic data. The physicochemical data of I obtained in this study is useful for the dosage form design of I.

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